DISSERTAÇÃO – ARTIGO DE INVESTIGAÇÃO MÉDICA

Mestrado Integrado em Medicina

BACTÉRIAS DORMENTES OBTIDAS DE BIOFILMES DE *STAPHYLOCOCCUS EPIDERMIDIS* TÊM MENOR POTENCIAL INFLAMATÓRIO E MANTÊM-SE TOLERANTES A VANCOMICINA E PENICILINA APÓS INICIAREM UM CRESCIMENTO PLANCTÓNICO

DORMANT BACTERIA WITHIN *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS HAVE LOW INFLAMMATORY PROPERTIES AND MAINTAIN TOLERANCE TO VANCOMYCIN AND PENICILLIN AFTER ENTERING PLANKTONIC GROWTH

José Filipe Dias Cerca

2014

DISSERTAÇÃO

BACTÉRIAS DORMENTES OBTIDAS DE BIOFILMES DE *STAPHYLOCOCCUS EPIDERMIDIS* TÊM MENOR POTENCIAL INFLAMATÓRIO E MANTÊM-SE TOLERANTES A VANCOMICINA E PENICILINA APÓS INICIAREM UM CRESCIMENTO PLANCTÓNICO

DORMANT BACTERIA WITHIN *STAPHYLOCOCCUS EPIDERMIDIS* BIOFILMS HAVE LOW INFLAMMATORY PROPERTIES AND MAINTAIN TOLERANCE TO VANCOMYCIN AND PENICILLIN AFTER ENTERING PLANKTONIC GROWTH

José Filipe Dias Cerca, Aluno do 6º ano profissionalizante do Mestrado Integrado em Medicina, Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto.

Orientador: Doutor Manuel Vilanova, Professor Associado, Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto.

O trabalho apresentado nesta dissertação foi realizado no Laboratório de Imunologia Mário Arala Chaves – ICBAS/UP. Actualmente encontra-se submetido para publicação na revista científica *Journal of Medical Microbiology* (Society for General Microbiology).

Dedicado a

Ana e Sofia

AGRADECIMENTOS

Ao Professor Doutor Manuel Vilanova, pelo empenho na orientação deste trabalho e pela sua preserverança no sentido de implementar o estudo de biofilmes de *Staphylococcus epidermidis* no laboratório de Imunologia Mário Arala Chaves.

Aos co-autores deste trabalho, pela apoio técnico e pelo sentido crítico na revisão deste manuscrito.

Aos elementos do Laboratório de Imunologia Mário Arala Chaves, pelo bom ambiente e disponibilidade para ajudar.

RESUMO

O *Staphylococcus epidermidis* (*S. epidermidis*) é uma bactéria Gram-positiva e coagulase-negativa sendo amplamente reconhecido como um comensal que coloniza o epitélio e mucosas humanas e que raramente é responsável por infecções em hospedeiros imunocompetentes. No entanto, nos últimos anos, a percepção médica de que esta bactéria seria uma espécie inócua foi-se alterando devido à sua capacidade para colonizar dispositivos médicos através da formação de biofilmes na superfície dos mesmos. Os biofilmes são definidos como uma comunidade de bactérias aderiras a uma superfície, que partilham uma arquitectura própria permanecendo embebidas por uma matriz extracelular, e que têm uma fisiologia/metabolismo distinto quando comparado com o seu estado livre/planctónico. Actualmente, dados epidemiológicos reconhecem o *S. epidermidis* como um dos principais agentes etiológicos de infecções nosocomiais. São exemplos de infecções por biofilmes de *S. epidermidis*: endocardites devido à colonização de válvula prostética; bacteriúria ou bacteriemia devido a colonização de cateteres urinários ou intra-vasculares respectivamente; endoftalmite devido a colonização de lente intra-ocular; ou infecção de prótese articular após artroplastia total do joelho ou anca. A elevada tolerância à antibioterapia que caracteriza os biofilmes determina que o tratamento destas infecções seja extremamente difícil, sendo muitas vezes necessária a remoção cirúrgica do dispositivo médico como método para eliminar o foco infeccioso. Assim, estas infecções associam-se frequentemente a elevada morbilidade para o doente assim como a elevados custos para o sistema de saúde.

O ciclo de formação de biofilmes de *S. epidermidis* envolve 3 etapas distintas: 1) a adesão inicial à superfície de contacto, um processo molecular mediado por diversos factores de aderência da bactéria assim como por interacções físicas/hidrofóbicas entre a bactéria e a superfície de contacto; 2) crescimento e maturação do biofilme, um processo onde ocorre agregação intercelular e é mediado pela síntese bacteriana de adesinas extracelulares sendo a mais conhecida a molécula poli-*N*-acetilglucosamina (PNAG); 3) o

estadio final do ciclo do biofilme envolve a libertação de bactérias para o meio circundante sendo que estas reiniciam novo crescimento na forma livre/planctónica. Esta etapa é considerada fundamental na patogénese dos biofilmes uma vez que constitui a base para a disseminação da infecção.

Neste trabalho de investigação pretendeu-se estudar as alterações fisiológicas que ocorrem em *S. epidermidis* durante a transição do crescimento em biofilme para o crescimento na forma livre/planctónica. Este trabalho inclui duas etapas principais: 1) uma primeira etapa em que validamos uma sonda fluorescente (SYBR green) como marcador para avaliar o estado fisiológico de bactérias *S. epidermidis*; 2) uma segunda etapa em que, usando essa mesma sonda fluorescente, fomos avaliar o comportamento de uma subpopulação de bactérias do biofilme (bactérias dormentes) na transição do crescimento em biofilme para o crescimento livre/planctónico. Para este efeito, e em termos de procedimentos experimentais, foram realizadas as seguintes técnicas: 1) crescimento *in vitro* de biofilmes de *S. epidermidis*, durante 48 horas a 37ºC com agitação constante de 80 rotações por minuto, usando meio de cultura enriquecido em glicose ou glicose com magnésio para condicionar as proporções de bactérias dormentes nos biofilmes; 2) citometria de fluxo para avaliar o estado fisiológico de bactérias *S. epidermidis* em diferentes intervalos de tempo mediante quantificação da intensidade de fluorescência emitida por bactérias marcadas com o fluorocromo SYBR green; 3) PCR em tempo real quantitativo para avaliar a expressão dos genes *icaA* e *rpiA* em diferentes intervalos de tempo; 4) avaliação da tolerância à vancomicina e penicilina nas diferentes culturas de *S. epidermidis* (biofilme, planctónicas e bactérias dormentes vs não dormentes) mediante quantificação da morte bacteriana por citometria de fluxo; 5) estabelecimento de culturas *in vitro* de células dendríticas derivadas da medula óssea e estimulação com inóculos obtidos de biofilmes de *S. epidermidis* com diferentes proporções de bactérias dormentes.

Para avaliar a transição do crescimento de *S. epidermidis* na forma de biofilme para a forma livre/planctónica, estabelecemos biofilmes de 48 horas com proporções reduzidas de bactérias dormentes. Destes biofilmes, uma série foi desagregada e as suspensões bacterianas resultantes foram colocadas a

crescer em meio fresco na forma livre/planctónica durante 6 horas. Paralelamente, e como controlo, outra série destes biofilmes permaneceu a crescer na forma de biofilme, com meio fresco, durante o mesmo intervalo de tempo. Em diferentes intervalos de tempo (0h, 1h30m, 3h e 6h) bactérias obtidas de ambas as culturas (biofilme e planctónica) foram analisadas por citometria de fluxo e a intensidade de fluorescência após marcação com o fluorocromo SYBR green foi quantificada. Verificou-se que, ao contrário das bactérias que permaneceram a crescer na forma biofilme, as bactérias que transitaram para um crescimento na forma livre/planctónica apresentaram um aumento progressivo da intensidade média de fluorescência, sendo o pico de intensidade de fluorescência detectado às 3 horas de crescimento.

Tendo em conta publicações anteriores que, usando estudos comparativos de análise transcriptomica entre biofilmes e células planctónicas, mostraram alterações distintas no padrão de expressão de genes entre bactérias crescidas no biofilme e na forma livre/planctónica, usamos PCR em tempo real para avaliar a expressão de dois genes que codificam proteínas envolvidas em vias metabólicas distintas do *S. epidermidis*: *icaA*, codifica uma proteína transferase envolvida na síntese de PNAG (maturação do biofilme); e *rpiA*, codifica uma isomerase de ribose-5-fosfato que participa na via redutora das pentoses fosfato com consequente geração de NADPH/energia. Verificou-se que as bactérias que se mantiveram a crescer na forma de biofilme expressaram o gene *icaA* em níveis significativamente maiores e o gene *rpiA* em níveis significativamente menores, quando comparadas com as bactérias que transitaram para o crescimento planctónico. Este resultado sugere que as bactérias que transitaram para o crescimento na forma planctónica usam a sua fonte de carbono para a produção de energia (geração de NADPH na via das pentoses fosfato), enquanto as bactérias que permanecem na forma de biofilme usam a fonte de carbono para a síntese de PNAG, molécula envolvida na adesão intercelular e maturação/crescimento do biofilme. Ao usar um método independente da citometria de fluxo para avaliar as diferenças no estado fisiológico de *S. epidermidis* crescido na forma de biofilme ou forma livre/planctónica, este resultado contribui para a validação do fluorocromo

SYBR green como sonda/marcador para avaliar o estado fisiológico desta bactéria.

A reversibilidade fisiológica entre bactérias que se encontram nos biofilmes e bactérias que iniciam um crescimento livre/planctónico tem sido referida em diversos estudos como uma importante característica para o seu perfil de tolerância/susceptibilidade a antibióticos. Tendo em conta este pressuposto, avaliou-se se a transição do crescimento de *S. epidermidis* em biofilme para culturas livres/planctónicas se associava a alterações no perfil de susceptibilidade à vancomicina e à penicilina. Para este efeito, culturas dos biofilmes e culturas planctónicas foram incubadas por um período de 30 minutos com vancomicina (40 ug/ml), penicilina (40 ug/ml) ou meio fresco (controlo negativo) e as proporções de bactérias mortas foram quantificadas por citometria de fluxo. Verificou-se que as bactérias em crescimento livre/planctónico eram significativamente mais susceptíveis a ambos os antibióticos, dado o aumento marcado de bactérias mortas que se detectou nas culturas planctónicas. Em oposição, nas culturas de biofilmes, a proporção de bactérias mortas após incubação com os antibióticos não aumentou em comparação com o grupo controlo (meio de cultura fresco sem antibiótico).

Assim, nesta primeira parte do trabalho, ao evidenciar que as alterações da intensidade de fluorescência devida à marcação com SYBR green durante a transição do crescimento em biofilme para o crescimento na forma livre/planctónica se associaram a alterações na expressão dos genes *icaA* e *rpiA* e alterações na sensibilidade aos antibióticos vancomicina e penicilina, reforçamos o potencial da utilização de SYBR green como sonda fluorescente para avaliar o estado fisiológico do *S. epidermidis*.

De seguida, definimos uma estratégia similar para avaliar as alterações fisiológicas que ocorrem em *S. epidermidis* durante a transição do crescimento em biofilme para o crescimento livre/planctónico mas, desta vez, focando a nossa atenção numa subpopulação de bactérias que ocorrem frequentemente nos biofilmes de *S. epidermidis*: as bactérias dormentes (ou não cultiváveis em meio sólido). Para esse efeito, e tendo em consideração estudos anteriores realizados no laboratório de Imunologia Mário Arala Chaves, usamos um meio

de cultura enriquecido em glucose mas sem suplementação de magnésio para estabelecer biofilmes de 48 horas com elevadas proporções de bactérias dormentes. Ao desagregar esses biofilmes e subsequentemente iniciar um crescimento planctónico verificou-se que uma grande proporção de bactérias manteve níveis de intensidade de fluorescência devida a SYBR green idênticos aos que apresentava enquanto se encontravam no biofilme. Assim, tendo em conta a intensidade de fluorescência de SYBR green, descriminamos duas populações de bactérias por citometria de fluxo: bactéria dormentes que mantiveram uma intensidade de fluorescência baixa de SYBR após iniciarem um crescimento planctónico; e bactérias não dormentes que, tal como mostrado anteriormente, apresentaram um aumento da intensidade de fluorescência com SYBR após iniciarem o crescimento planctónico. Avaliou-se também, de forma idêntica ao descrito anteriormente, a susceptibilidade dessas populações bacterianas à vancomicina e à penicilina. Verificou-se que as bactérias dormentes não alteraram o seu estado fisiológico após iniciarem o crescimento planctónico e mantiveram uma elevada tolerância a estes antibióticos. Já as bactérias não dormentes, que alteram o estado fisiológico após iniciarem o crescimento planctónico, tornaram-se susceptíveis a acção destes antibióticos. Estes resultados demonstraram que as bactérias dormentes dos biofilmes de *S. epidermidis* não alteram o seu estado fisiológico ao iniciar um crescimento planctónico mantendo a tolerância à vancomicina e à penicilina.

Por fim, exploramos a interacção *in vitro* de células dendríticas derivadas da medula óssea com bactérias obtidas de biofilmes de *S. epidermidis* com elevadas ou reduzidas proporções de bactérias dormentes. A activação das células dendríticas foi avaliada mediante quantificação das citocinas pro-inflamatórias TNF-α, IL-12 e IL-6 nos sobrenadantes das culturas. Verificou-se que o inoculo de bactérias obtido a partir de biofilmes com maiores proporções de bactérias dormentes induziu uma menor produção de TNF-α, IL-12 e IL-6, sugerindo uma interacção diferenciada destas bactérias com componentes do sistema imune inato do hospedeiro.

Em resumo, mostramos que o fluorocromo SYBR green pode ser usado como uma sonda/marcador fluorescente para avaliar por citometria de fluxo o estado fisiológico do *S. epidermidis* quando crescido na forma de biofilme ou na forma livre/planctónica e demonstramos que a subpopulação de bactérias dormentes presente nos biofilmes de *S. epidermidis* não altera o seu estado fisiológico quando inicia um crescimento planctónico assim como mantém a tolerância à vancomicina e à penicilina. Estes resultados realçam que os biofilmes, já intrinsecamente tolerantes aos antibióticos, podem libertar bactérias para o meio ambiente que mantêm um perfil de tolerância aos antibióticos o que, por sua vez, lhes pode conferir maior capacidade para persistir no hospedeiro. Considerando que os biofilmes presentes nos dispositivos médicos são referidos na literatura como apresentando consistentemente uma elevada frequência de bactérias dormentes, estudos futuros que abordem o papel das bactérias dormentes nas infecções de dispositivos médicos por biofilmes de *S. epidermidis* poderão contribuir para aumentar a compreensão da fisiopatologia deste agente etiológico nosocomial.

Dormant bacteria within *Staphylococcus epidermidis* biofilms have low inflammatory properties and maintain tolerance to vancomycin and penicillin after entering planktonic growth

Filipe Cerca[a,b], Ângela França[c], Begoña Pérez-Cabezas[a,b], Adília Ribeiro[a,b], Joana Azeredo[c], Gerald Pier[f], Nuno Cerca[c], Manuel Vilanova[a,b,*]

[a] ICBAS-UP - Instituto de Ciências Biomédicas de Abel Salazar - Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313, 4099-003, Porto, Portugal;

[b] IBMC - Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 83, Porto, Portugal;

[c] CEB-IBB, Centro de Engenharia Biológica - Instituto de Biotecnologia e Bioengenharia, Campus de Gualtar, Universidade do Minho, Braga, Portugal;

[d] UMIB – Unidade Multidisciplinar de Investigação Biomédica, Universidade do Porto, Largo Prof. Abel Salazar 2, 4099-003, Porto, Portugal;

[e] Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital/Harvard Medical School, Boston, MA 02115, USA

Running title: *S. epidermidis* dormant cells

*** Corresponding author:**
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto, Portugal
Tel: 351-220428214
E-mail: vilanova@icbas.up.pt

Abstract

Staphylococcus epidermidis is the most commonly isolated etiological agent of nosocomial infections mainly due to its ability to establish biofilms on indwelling medical devices. Detachment of bacteria from *S. epidermidis* biofilms and subsequent growth in the planktonic form is a hallmark in the pathogenesis of these infections leading to dissemination. Here we showed that *S. epidermidis* cells collected from biofilms cultured in conditions that promote cell viability present marked changes in their physiological status upon initiating a planktonic mode of growth. When compared to cells growing in biofilms, they displayed an increased SYBR green I staining intensity, increased transcription of the *rpiA* gene, decreased transcription of *icaA* gene as well as higher susceptibility to vancomycin and penicillin antibiotics. When bacteria collected from biofilms with high proportions of dormant cells were subsequently cultured in the planktonic mode, a large proportion of cells maintained a low SYBR staining intensity and increased resistance to vancomycin and penicillin, a profile typical of dormant cells. This phenotype further associated with a decreased ability of these biofilm-derived cells to activate bone marrow-derived dendritic cells in vitro, as determined by pro-inflammatory cytokine quantification. These results demonstrated that cells detached from the biofilm maintain a dormant cell-like phenotype, having a low pro-inflammatory effect and decreased susceptibility to antibiotics suggesting these cells may contribute for the recalcitrant nature of biofilm infections.

Keywords: biofilms; *S. epidermidis*; dormant bacteria; pathophysiology; antibiotic tolerance, penicillin, vancomycin, dendritic cells

Introduction

Staphylococcus epidermidis is a commensal bacterium that colonizes the skin and mucous membranes, often being the most prevalent staphylococcal species found on human skin (Otto, 2009). The ability to colonize and establish biofilms on indwelling medical devices makes this bacterium the most commonly isolated etiological agent of nosocomial infections (Uçkay et al., 2009). Moreover, due to the intrinsic resistance of staphylococcal biofilms to antibiotics (Raad et al., 1998), staphylococcal biofilm-originated infections are associated with an increased duration of hospital admissions and use of medical resources and, consequently, of healthcare costs (Dimick et al. 2001; Rogers et al., 2009). Critically-ill immune-compromised patients (Bearman & Wenzel, 2005) and premature neonates (Fallat et al., 1998) are the individuals most vulnerable to this opportunistic pathogen.

S. epidermidis biofilm formation involves initial cellular adherence to a surface followed by intercellular aggregation and accumulation in multilayered cell clusters (Otto, 2009). This process is dependent on the synthesis of adhesive extracellular molecules (Götz, 2002), such as the polysaccharide intercellular adhesin (PIA) also known as poly-*N*-acetyl glucosamine (PNAG), a major constituent mediating cell-to-cell adhesion in staphylococci (Mack et al., 1996; Mack et al., 1994). The final stage of the biofilm life cycle comprises cell detachment and subsequent growth in the planktonic form, a process that is crucial for *S. epidermidis* biofilm pathogenesis by forming the basis for dissemination of infection (Wang et al., 2011). Therefore, in this study we evaluated physiological changes occurring in *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth. We show that dormant bacteria within *S. epidermidis* biofilms display a low inflammatory profile and increased tolerance to vancomycin and penicillin upon initiating a planktonic growth.

Material and Methods

Bacterial strains and growth conditions

S. epidermidis strain 9142 (Nedelmann et al., 1998) was used in this study. To establish a 48 h biofilm, a starter culture was grown overnight in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) at 37ºC with agitation (80 rpm). The optical density at 640 nm of the starter culture was adjusted to 0.250 with phosphate buffered saline (PBS) and a 10 µL aliquot transferred to a 24-well polystyrene plate (Nunc, Roskilde, Denmark) containing 1 mL/well of TSB with 0.4% filtered glucose (w/v) (Merck), further supplemented with 10 mM $MgCl_2$ (Merck). The plates were then incubated for 48 h at 37ºC with agitation (80 rpm). At 24 h of growth, the culture medium was replaced by fresh TSB supplemented with 1% of glucose and 10 mM $MgCl_2$ (TSB 1%G + Mg^{2+}). Similar culture conditions were used to establish 48 h biofilms with high proportions of dormant bacteria by using growth medium without $MgCl_2$ supplementation, as previously described (Cerca et al., 2011a).

Preparation of biofilm and planktonic *S. epidermidis* cell suspensions

To assess the bacterial physiological status during the transition from the biofilm to the planktonic mode of growth, 48 h biofilms grown in TSB 1%G + Mg^{2+} were disaggregated as previously described (Cerca et al., 2011b) and the resulting cell suspensions were diluted in fresh TSB to a concentration of 1×10^8 cells/mL. These cell suspensions were allowed to grow in the planktonic form for a 6 h period (37ºC, 80 rpm). Simultaneously, the culture medium of 48 h biofilm cultures was washed out and biofilms were allowed to grow for an additional 6 h period in 1 mL fresh TSB. At the time points 0 h, 1 h 30 min, 3 h and 6 h of growth, an aliquot of bacteria was recovered from each culture (biofilm and planktonic) and used for flow cytometry analysis and gene expression quantification, as described below.

Flow cytometry analysis

At the indicated time points, biofilms were washed twice with 1 mL of PBS, and bacteria were then recovered in 1 mL of PBS, as previously described (Cerca et al., 2011b). After a 1:10 dilution in PBS, an aliquot of 30 μL was transferred to 270 μL of PBS containing 3 μL of quantification microspheres (Invitrogen, Carlsbad, CA, USA), SYBR green I (SYBR, Invitrogen) (1:5000 commercial stock) and propidium iodide 5 μg/mL (PI, Sigma, St. Louis, MO, USA). For the planktonic cultures, an aliquot of 30 μL of cells was transferred from the culture to 270 μL of PBS containing 3 μL of quantification microspheres (Invitrogen), SYBR (1:5000 commercial stock) and PI 5 μg/mL. Bacterial fluorescence analysis was carried out by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) containing a low-power air-cooled 15 mW blue (488 nm) argon laser. Data were acquired using the CellQuest software (Becton Dickinson) and analyzed using the Flowjo 7.2.5 software (Tree Star, Ashland, OR, USA). SYBR fluorescence was detected on the FL1 channel (BP530/30) while PI fluorescence was detected on the FL3 channel (LP650). For all detected parameters, amplification was carried out using logarithmic scales. The concentration of bacteria in the planktonic or biofilm cultures was further determined by acquiring the counts for a specific number of microspheres during flow cytometry analysis of the cell samples.

Quantitative PCR

Quantitative PCR (qPCR) was used to assess the expression of intercellular adhesin A (*icaA*) and ribose-5-phosphate isomerase A (*rpiA*) genes in *S. epidermidis* bacteria grown in biofilm or planktonic cultures. The primers used were designed with the Primer3 software (Rozen and Skaletsky, 2000) using the *S. epidermidis* RP62A genome as a template (PubMed NC_002976.3). The sequences of the primers used are listed in Table 1. Primer efficiency was determined by the dilution method and by performing a temperature gradient reaction from 54 to 64ºCThe set of primers having the optimal and most similar efficiency values at 60ºC were used. At each time point, total RNA from either biofilm or planktonic cultures was extracted using the FastRNA® Pro Blue Kit

(MP Biomedicals, Solon, OH, USA), as described previously (França et al., 2011). Contaminating genomic DNA was removed by treatment with DNase I (Fermentas, Burlington, ON, Canada) for 30 min at 37ºC. The enzyme was then heat-inactivated at 65ºC for 10 min in the presence of EDTA. Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at –80ºC. Total RNA samples were reverse transcribed in the presence of each reverse primer and RevertAid^{TM} M-MuLV Reverse Transcriptase (Fermentas). Control reactions lacking the reverse transcriptase enzyme (no-RT) were included. qPCR reactions contained 2 µL 1:200 diluted cDNA or no-RT control, 2 µL containing 10 pmol of each primer, 6 µL nuclease free deionized H_2O, and 10 µL Maxima® SYBR Green qPCR Master Mix (Fermentas) with the following thermocycler parameters: 94ºC for 10 min, 40 cycles of 94ºC for 15 s, 60^{o}C for 20 s and finally 72ºC for 25 s. To monitor the reaction specificity and primer dimer formation, end-products were analyzed by melting curves. Relative fold increase was calculated using $2^{\Delta Ct}$ method, a variation of the Livak method, where ΔCt = Ct (housekeeping gene)-Ct (target gene). The data analysis was based on at least 2 independent experiments.

Evaluation of the susceptibility to vancomycin and penicillin of *S. epidermidis* biofilm bacteria after initiating a planktonic growth

S. epidermidis bacteria obtained from 48 h biofilms grown in TSB 1%G + Mg^{2+} were diluted in fresh TSB (1×10^8 cells/mL) and allowed to grow in the planktonic form for a 3 h period. Simultaneously, parallel cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 3 h period in fresh TSB. At this time point, vancomycin (40 µg/mL) (Sigma), penicillin (40 µg/mL) (Sigma) or TSB (negative control) were added to the biofilm and planktonic cultures during a 30 min period. Bacterial death was determined through flow cytometry by assessing the bacterial incorporation of PI, as described above. A similar procedure was done using 48 h biofilms grown in TSB 1%G without magnesium supplementation as a starting point.

Mice

Male BALB/c mice 6–8 weeks of age were purchased from Charles River and kept at the animal facilities of the Institute Abel Salazar (ICBAS, Portugal) during the experiments. Hiding and nesting materials were provided for enrichment. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued by the competent national board (Direção Geral de Veterinária), document number 0420/000/000/2008.

Bone marrow-derived dendritic cells (BMDCs) differentiation

Bone marrow cells were collected from femurs and tibias of BALB/c mice by flushing with cold RPMI 1640 (Sigma). Cells (1×10^6/mL) were cultured in 6-well plates in RPMI supplemented with 15% (v/v) J558-cell supernatant, 10% FBS (PAA), penicillin (100 U.I./mL)-streptomycin (100 µg/mL) (Sigma), and L-Glutamine (2mM) (Sigma) and incubated at 37ºC, 5% CO2. Half of the media was renewed every two days. At day 6, BMDCs were detached, adjusted at a concentration of 1×10^6/mL, distributed in 96-well round bottom plates (100 µL per well) and incubated overnight in supplemented RPMI medium.

Stimulation of BMDCs with bacterial suspensions of *S. epidermidis* obtained from different cultures

Bacterial suspensions were obtained from 48 h biofilms grown in TSB 1%G or TSB 1%G + Mg^{2+} as described above. For planktonic cultures, a single colony of *S. epidermidis* was inoculated into 35 mL TSB and grown overnight at 37ºC in a shaker rotator at 80 rpm. Then, cells were recovered, centrifuged during 10 min at 13000 rpm at 4ºC (Biofuge fresco HERAEUS, ThermoSCIENTIFIC, Waltham, MA, USA), and resuspended in 1mL of PBS. Before stimulation of BMDCs, all the bacterial inocula were resuspended in RPMI supplemented with 10% FBS and 2mM L-glutamine and adjusted to the concentration of 1×10^6 cells / mL. BMDCs were then stimulated with 100 µL of bacterial suspensions

(1×10^6/mL) obtained from biofilms grown in TSB 1%G, TSB 1%G + Mg^{2+} or original planktonic cultures. RPMI supplemented media and lipopolysaccharide (LPS, 1µg/mL) (Sigma) were used as negative and positive controls, respectively. After 6h of incubation (37ºC, 5% CO_2), media containing bacteria was collected and replaced by fresh medium containing penicillin (200 U.I./mL) and streptomycin (200 µg/mL). At the 6 h or 18 h time points, the culture supernatants were removed and stored at -20 ºC until use.

Cytokine quantification

Interleukin (IL) 6, IL-12p70 and tumor necrosis factor (TNF-α) were quantified using commercially available quantification kits (eBioscience Inc, San Diego, CA, USA) according to the manufacturer's instructions. Results were read in a Multiskan Ex spectrophotometer (Thermo Electron Corporation, Corston, UK) using the Ascent software (Thermo Electron Corporation).

Statistical analysis

All graphs were generated using GraphPad Prism software (GraphPad Software). Means and standard errors of the means (SEM) were calculated. Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests or with one-way analysis of variance with Tukey's Multiple Comparison Test. Both tests used GraphPad software. A P value of <0.05 was considered statistically significant.

Results and Discussion

S. epidermidis cells that undergo a shift from biofilm to planktonic growth present a high SYBR staining intensity and *rpiA* gene expression

Detachment of bacteria from a biofilm and subsequent growth in the planktonic form is considered a major event in the pathophysiology of biofilm related infections (Otto, 2013). In *S. epidermidis* this was previously shown to be followed by a transition from a non-aggressive, non-growing and fermentative state (biofilm stage of growth) into a growing, aggressive/inflammatory and respiratory state (planktonic stage of growth) (Yao et al., 2005). SYBR staining intensity was previously found to correlate with *S. epidermidis* respiratory activity, as demonstrated by co-staining studies using SYBR and the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Cerca et al., 2011c). Therefore, we used SYBR as a fluorescent probe to evaluate physiological changes of *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth. For this purpose, 48 h biofilms grown in TSB media supplemented with glucose and magnesium (TSB 1%G + Mg2+) were prepared and used as the starting point of our study, as these conditions maintain the majority of cells in a culturable state (Cerca et al., 2011a). As shown in Figure 1, bacterial suspensions were prepared from the 48 h biofilms and allowed to grow in the planktonic form for a further 6 h. Simultaneously, parallel cultures of *S. epidermidis* biofilms continued growing for an additional 6 h in fresh TSB medium. Bacterial cells were then obtained at different time points from either the planktonic or biofilm cultures, stained with SYBR and PI, and analysed by flow cytometry. The bacteria that entered into the planktonic growth phase progressive gained increases in the mean fluorescence intensity (MFI) due to SYBR staining, reaching a detected maximum at the 3 h time point (Figure 1). In contrast, bacteria that remained growing in the biofilm mode presented a lower and constant SYBR MFI over time (Figure 1). Our results are in agreement with a previous report showing that the shift from the biofilm to the planktonic mode of growth is accompanied by an increase in the *S. epidermidis* respiratory activity (Yao et al., 2005). Interestingly, an increase in PI staining

intensity was detected in the bacteria obtained from the planktonic cultures at the 90 min time point that was not detected on the two later assessed time points. Accordingly to previous reports, this transient PI-incorporating state may correspond to temporally-compromised cell membrane integrity due to a fast increase in cell size (Lybarger & Maddock, 2001; Shi et al., 2007).

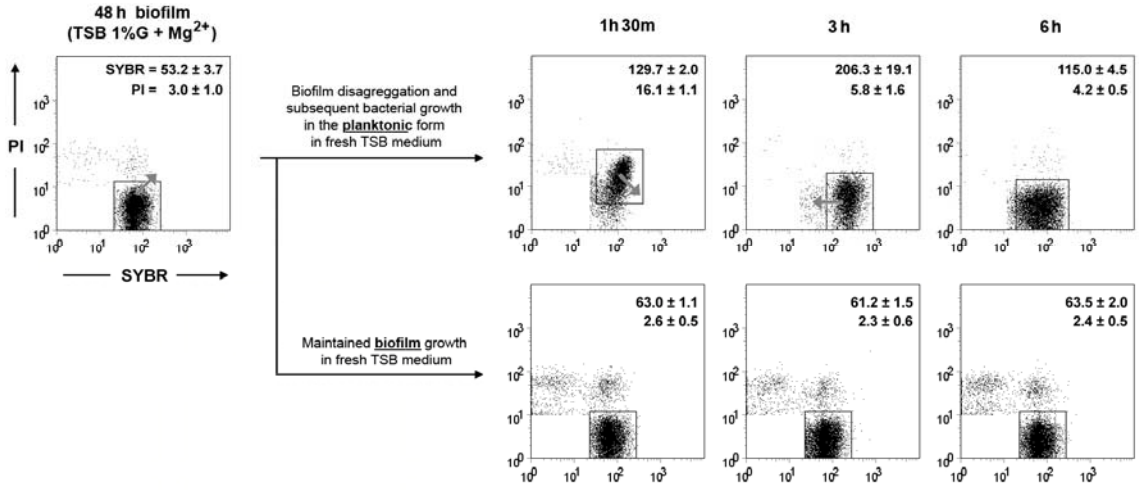

Figure 1. Evaluation of SYBR staining intensity in *S. epidermidis* bacteria upon the shift from the biofilm to the planktonic mode of growth. Bacteria obtained from low dormancy 48 h biofilms were diluted in fresh TSB (1×10^8 cells/mL) and allowed to grow in the planktonic form for 6 h. Simultaneously, parallel cultures of 48 h biofilms were allowed to grow for an additional 6 h in fresh TSB. At the indicated time points, bacteria obtained from each culture were stained with SYBR and PI (5 µg/mL) and the bacterial fluorescence determined by flow cytometry analysis. Values within the bi-parametric dot plots (SYBR vs PI) represent the mean fluorescence intensity (MFI) of the SYBR- or PI- signal ± standard deviation (SD) from bacteria within the respective flow cytometry analysis region. Arrows within the dot plots represent the direction of the shift in the bacterial MFI due to SYBR/PI staining between assessed time points. Results are a representative example of two independent experiments that generated concordant results.

A previous study in which a comparative transcriptome analysis in biofilm and planktonic *S. aureus* cells was carried out showed that planktonic bacteria presented an increased respiratory activity and decreased synthesis of poly-*N*-acetylglucosamine as compared with their biofilm counterparts (Resch et al., 2005). Therefore, in order to further characterize the physiological alterations

occurring in *S. epidermidis* bacteria during the transition from the biofilm to the planktonic mode of growth, we evaluated the transcription of *icaA* and *rpiA* mRNA in bacteria obtained from the biofilm or planktonic cultures at different time points. The *icaA* gene encodes a *N*-acetyl-glucosamine transferase involved in the synthesis of PIA (Heilmann et al., 1996) whereas *rpiA* encodes a ribose-5-phosphate isomerase that participates in the NADPH-generating pentose phosphate pathway (Jeppsson et al., 2002).

Table I. Oligonucleotide sequence used for qPCR.

Target gene		Oligonucleotide primers sequence (5' to 3')	TM (^{o}C)	Amplicon size (bp)
16S	FW	GGGCTACACACGTGCTACAA	59.8	176
	RV	GTACAAGACCCGGGAACGTA	59.9	
icaA	FW	TGCACTCAATGAGGGAATCA	60.2	134
	RV	TAACTGCGCCTAATTTTGGATT	59.9	
rpiA	FW	CAACAACGACAAATCGGTCA	60.5	114
	RV	CAATAGATGGCGCTGATGAA	59.8	

As shown in Figure 2A, bacteria that remained in the biofilm cultures expressed, over time, significantly higher levels of *icaA* than bacteria that initiated the planktonic growth. In contrast, planktonic bacteria expressed significantly higher levels of *rpiA* than their biofilm counterparts (Figure 2B). These results by showing a correlation between SYBR^{high} staining intensity and increased *rpiA* expression provide additional evidence for the suitability of using this fluorescent dye as a probe to evaluate the respiratory status of *S. epidermidis* cells.

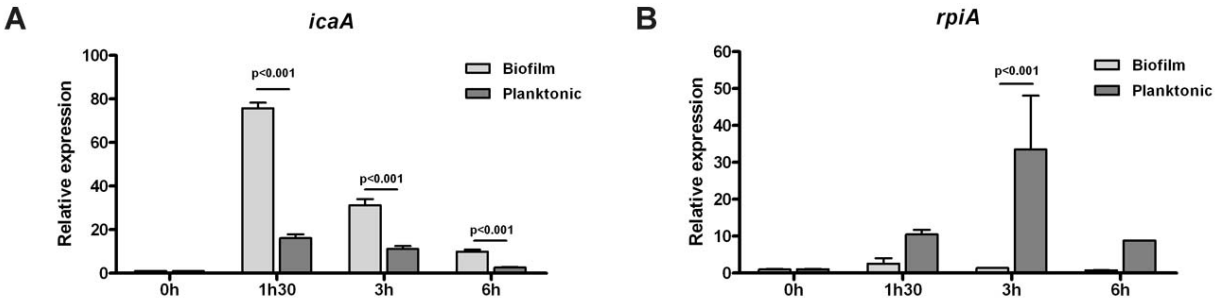

Figure 2. Evaluation of *icaA* and *rpiA* gene expression over time in *S. epidermidis* bacteria grown in the biofilm or planktonic modes. Bacteria obtained from low dormancy 48 h *S. epidermidis* biofilms were diluted in fresh TSB (1×10^8 cells/mL) and allowed to grow in the planktonic form for a 6 h period. Simultaneously, parallel cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 6 h period in fresh TSB. At the indicated time points, a sample of bacteria was obtained from each culture and the expression of A) *icaA* and B) *rpiA* genes was evaluated by qPCR. Results shown are representative of two independent experiments that generated concordant results. Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests.

S. epidermidis biofilm bacteria display a higher susceptibility to vancomycin and penicillin after initiating a planktonic growth

The physiological reversibility between biofilm and planktonic bacteria has been pointed as an important determinant in their antibiotic- tolerant or susceptible profile (Fux et al., 2005). Since we showed that marked physiological alterations occurred in *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth, we evaluated whether this event was also accompanied by alterations in the susceptibility to vancomycin and penicillin. Attending that the major differences between biofilm and planktonic cells regarding SYBR MFI and rpiA expression were detected at 3 h (Figure 1 and 2), we selected this time point to assess the susceptibility of biofilm and planktonic bacteria to the above mentioned antibiotics. For this purpose, vancomycin (40 µg/mL), penicillin (40 µg/mL) or TSB (negative control) were added to the biofilm and planktonic cultures during a further 30 min. Bacterial death was then quantified by PI incorporation, detected by flow cytometry. As shown in Figure 3, bacteria within the planktonic cultures were more susceptible to vancomycin or penicillin as evaluated by the marked increase in the proportions of dead bacteria. In contrast, bacteria that remained growing in the

biofilm cultures maintained tolerance to vancomycin and penicillin as indicated by the lack of increased proportions of dead cells in these cultures. This result is in agreement with previous reports highlighting the role of the bacterial physiological status in determining antibiotic tolerance/susceptibility (Fux et al., 2005).

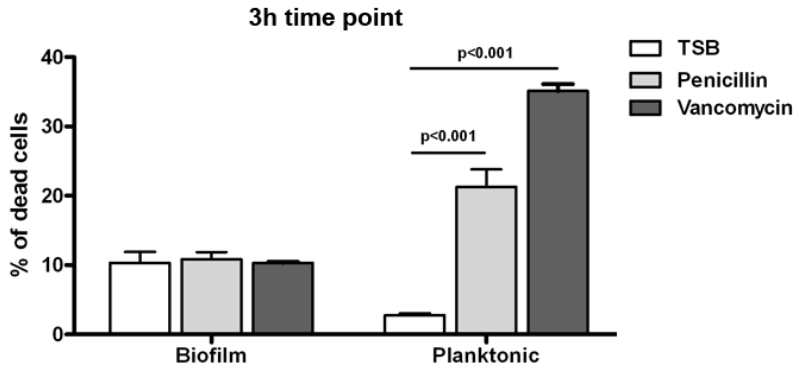

Figure 3. *S. epidermidis* biofilm bacteria acquire susceptibility to vancomycin and penicillin after initiating a planktonic growth. Bacteria obtained from low dormancy 48 h *S. epidermidis* biofilms were diluted in fresh TSB (1×10^8 cells/mL) and allowed to grow in the planktonic form for a 3 h period. Simultaneously, parallel cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 3 h period in fresh TSB. At this time point, vancomycin (40 μg/ml), penicillin (40 μg/ml) or TSB (negative control) were added to the biofilm and planktonic cultures that were grown for an additional 30 min. Bacterial death was determined by flow cytometry by assessing the bacterial incorporation of PI. Results shown are representative of two independent experiments that generated concordant results. Statistical analysis was carried out by two-way repeated-measures analysis of variance (ANOVA) with Bonferroni post tests.

Dormant bacteria within *S. epidermidis* biofilms maintain tolerance to vancomycin and penicillin when cultured in planktonic conditions

It is well established that the majority of bacteria within infectious biofilms may not grow under standard laboratory culture conditions and are in an apparent dormant state (Costerton et al., 2011; Oliver, 2010). This finding raises the question as to whether dormant bacteria within biofilms would also undergo a physiological shift when placed in planktonic culture conditions. To evaluate

this, we established 48 h biofilms with high proportions of dormant bacteria by using TSB+1%G without magnesium supplementation (Cerca et al., 2011a).

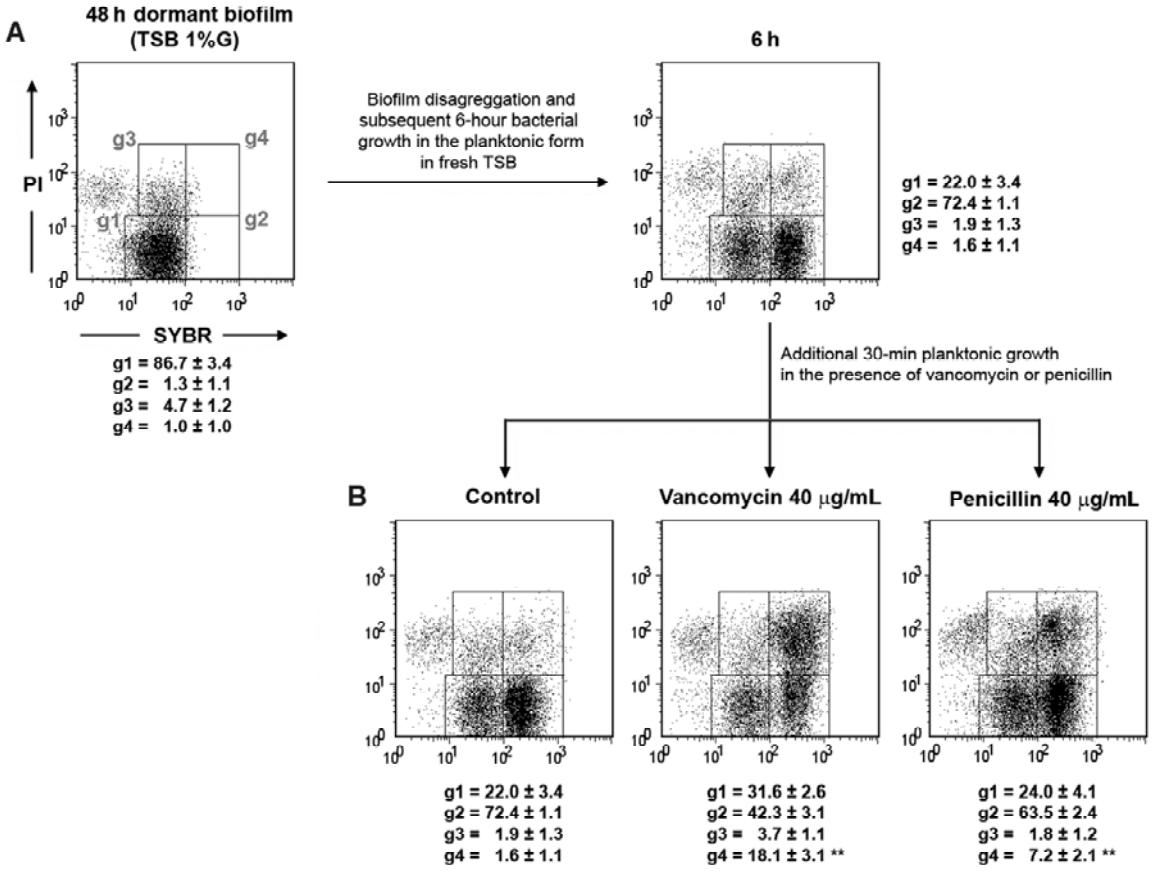

Figure 4. Dormant bacteria within *S. epidermidis* biofilms maintain tolerance to vancomycin and penicillin after initiating a planktonic growth. A) Cell suspensions obtained from biofilms with high proportions of dormant bacteria were diluted in fresh TSB (1×10^8 cells/mL) and allowed to grow a further 6 h period in the planktonic form. At this time point, the bacterial SYBR staining intensity was determined by FACS analysis. **B)** Vancomycin (40 μg/mL), penicillin (40 μg/mL) or TSB were added to the 6 hour planktonic cultures for a period of 30 minutes. Bacterial death was determined by flow cytometry analysis assessing incorporation of PI. Results shown are representative of two independent experiments that generated concordant results. Statistical significant differences in the proportions of $SYBR^{high}PI^{+}$ bacteria (gate g4) between control, vancomycin and penicillin groups ($p<0.01$, ANOVA) are indicated by asterisks (**). No statically significant differences were found in the proportions of $SYBR^{low}PI^{+}$ bacteria (gate g3) between control, vancomycin and penicillin groups.

As shown in Figure 4A, 6 h after disaggregation of the biofilms and transfer of biofilm-cells into planktonic culture conditions, the majority of bacteria displayed a $SYBR^{high}$ staining profile (gate g2), previously shown to correspond to growing non-dormant bacteria (Cerca et al., 2011c). However, a noticeable proportion of bacteria maintained a $SYBR^{low}$ staining intensity (gate g1), corresponding to dormant bacteria. As dormant and non-dormant bacteria could be discriminated by using flow cytometry, we further characterized these bacterial populations regarding susceptibility to vancomycin and penicillin. Thus, vancomycin (40 µg/mL), penicillin (40 µg/mL) or TSB (negative control) where added to the 6 h planktonic cultures during a 30 min period, upon which bacterial death was similarly determined by assessing PI incorporation. As shown in Figure 4B, no significant increase in the proportions of $SYBR^{low}PI^{+}$ cells (gate g3) was detected upon addition of antibiotics as compared with the control cultures. In contrast, bacteria that underwent a physiological shift, presenting a $SYBR^{high}$ staining (gate g4) were more susceptible to these antibiotics, as determined by the marked increase in the proportions of $SYBR^{high}PI^{+}$ cells. Altogether these results indicate that dormant bacteria within *S. epidermidis* biofilms do not undergo a physiological shift upon placed in planktonic culture conditions maintaining instead a low SYBR staining intensity that associated with tolerance to vancomycin and penicillin.

S. epidermidis **bacteria obtained from biofilms with high proportions of dormant cells induce a low activation of murine BMDC *in vitro***

As we determined that dormant bacteria within *S. epidermidis* biofilms presented a particular phenotype by maintaining tolerance to vancomycin and penicillin after initiating a planktonic growth, we further explored whether these cells interacted differently with murine BMDC compared to cells obtained from biofilms with lower proportions of dormant cells. Bacterial suspensions were prepared with cells obtained from high dormancy biofilms (TSB 1%G), low dormancy biofilms (TSB 1%G + Mg2+) or planktonic cultures and used to stimulate BMDC in vitro. BMDC activation was assessed by quantification of the proinflammatory cytokines TNF-α, IL-12 and IL-6 in the culture supernatants.

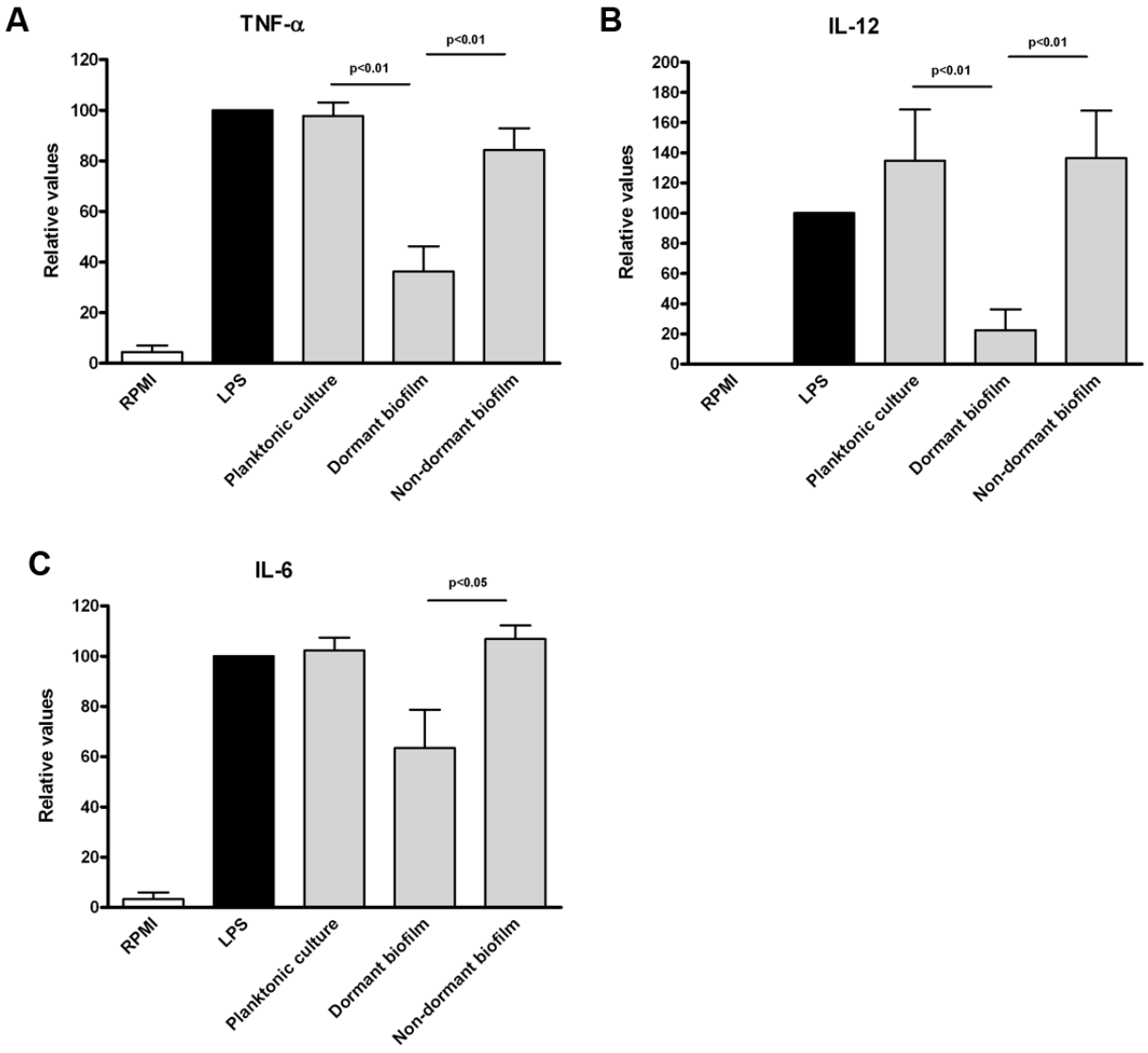

Figure 5. *S. epidermidis* bacteria obtained from high dormancy biofilms induce a lower activation of murine BMDC. Cell suspensions obtained from 48 h biofilms grown in TSB 1%G (Dormant biofilms), 48 h biofilms grown in TSB 1%G + Mg^{2+} (Non-dormant biofilms) or original planktonic cultures (Planktonic) were used to stimulate bone marrow-derived dendritic cells (BMDCs) *in vitro*. Activation of BMDCs was determined by the quantification of the proinflammatory cytokines **A)** tumor necrosis factor α (TNF-α), **B)** interleukin (IL) -12 and **C)** IL-6 in the cultures supernatants. Results shown are representative of two independent experiments that generated concordant results. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test.

As shown in Figure 5, bacteria obtained from biofilms with higher proportions of dormant cells induced the lower production of TNF-α, IL-12 and IL-6. Noteworthy, bacteria obtained from low dormancy biofilms activated

BMDC to the same extent as bacteria obtained from planktonic cultures. These results are in agreement with our previous report showing that *S. epidermidis* bacteria obtained from biofilms enriched in dormant cells induced a low activation of murine macrophages *in vitro* and *in vivo* (Cerca et al., 2011a). Also in agreement, a previous study have shown that staphylococcal biofilms could attenuate the inflammatory response of murine macrophages, as compared to planktonic cell counterparts, by promoting differentiation of these host cells into a M2 phenotype (Thurlow et al., 2011). It would thus be interesting to ascertain in future studies whether or to what extent dormant cells may contribute to this differential role of biofilm cells on host mononuclear phagocytes.

Concluding remarks

The physiological shift that occurs in *S. epidermidis* bacteria during the transition from the biofilm to the planktonic mode of growth is considered a major event in the pathophysiology of this bacterium (Yao et al., 2005). Here, we showed that bacteria from biofilms grown in conditions that promoted cell viability displayed increased SYBR staining intensity and *rpiA* gene expression as well as increased susceptibility to vancomycin and penicillin. In contrast, we also showed that a high proportion of bacteria obtained from biofilms grown in high glucose, a condition promoting dormancy, maintained a low SYBR staining intensity and tolerance to vancomycin and penicillin upon initiating a planktonic growth. These data highlight that biofilms, already intrinsically tolerant to antibiotics (Høiby et al., 2010), may release cells to the surrounding environment that maintain an antibiotic-tolerant profile. This, in turn, may confer on these bacteria an increased likelihood to persist within a host and cause disease, which would be in agreement with a recent study reporting an association between the clinical symptoms of biofilm-related infections and the presence of unculturable bacteria within the biofilms (Zandri et al., 2012). As infectious biofilms may have a high frequency and prevalence of unculturable bacteria (Oliver, 2010), further studies addressing the role of dormant bacteria in the pathophysiology of *S. epidermidis* biofilms would help understand the clinical outcomes of biofilm-related infections.

Acknowledgments

This work was funded by Fundação para a Ciência e a Tecnologia (FCT) and COMPETE grants PTDC/BIA-MIC/113450/2009 and FCOMP-01-0124-FEDER-014309. Parts of the work were also supported by National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIAID) grants AI46706 and AI057159, a component of Award Number U54 AI057159. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. The authors acknowledge the technical assistance of Encarnação Rebelo.

References

Bearman, G.M.L., Wenzel, R.P., 2005. Bacteremias: A Leading Cause of Death. Arch Med Res 36, 646-659.

Cerca, F., Andrade, F., Franca, A., Andrade, E., Ribeiro, A., Almeida, A., Cerca, N., Pier, G., Azeredo, J., Vilanova, M., 2011a. *Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. J Med Microbiol 60, 1717-1724.

Cerca, F., França, Â., Guimarães, R., Hinzmann, M., Cerca, N., Lobo da Cunha, A., Azeredo, J., Vilanova, M., 2011b. Modulation of poly-*N*-acetylglucosamine accumulation within mature *Staphylococcus epidermidis* biofilms grown in excess glucose. Microbiol Immunol 55, 673-682.

Cerca, F., Trigo, G., Correia, A., Cerca, N., Azeredo, J., Vilanova, M., 2011c. SYBR green as a fluorescent probe to evaluate the biofilm physiological state of *Staphylococcus epidermidis*, using flow cytometry. Can J Microbiol 57, 850-856.

Cerca, N., Martins, S., Cerca, F., Jefferson, K.K., Pier, G.B., Oliveira, R., Azeredo, J., 2005. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antimicrob Chemother 56, 331-336.

Costerton, J.W., Post, J.C., Ehrlich, G.D., Hu, F.Z., Kreft, R., Nistico, L., Kathju, S., Stoodley, P., Hall-Stoodley, L., Maale, G., James, G., Sotereanos, N., DeMeo, P., 2011. New methods for the detection of orthopedic and other biofilm infections. FEMS Immunol Med Microbiol 61, 133-140.

Dimick J.B., Pelz, R.K., Consunji, R., Swoboda, S.M., Hendrix, C.W., Lipsett P.A., 2001. Increased resource use associated with catheter-related bloodstream infection in the surgical intensive care unit. Arch Surg 136, 229-234.

Fallat, M., Gallinaro, R., Stover, B., Wilkerson, S., Goldsmith, L., 1998. Central venous catheter bloodstream infections in th neonatal intensive care unit. J Pediatr Surg 33, 1383-1387.

França, A., Melo, L., Cerca, N., 2011. Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis*. BMC Res Notes 4, 572.

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P., 2005. Survival strategies of infectious biofilms. Trends Microbiol 13, 34-40.

Götz, F., 2002. Staphylococcus and biofilms. Mol Microbiol 43, 1367-1378.

Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D., Götz, F., 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol 20, 1083-1091.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms. International journal of antimicrobial agents 35, 322-332.

Jeppsson, M., Johansson, B., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F., 2002. Reduced Oxidative Pentose Phosphate Pathway Flux in Recombinant Xylose-Utilizing *Saccharomyces cerevisiae* Strains Improves the Ethanol Yield from Xylose. Appl Environ Microbiol 68, 1604-1609.

Lybarger, S.R., Maddock, J.R., 2001. Polarity in Action: Asymmetric Protein Localization in Bacteria. J Bacteriol 183, 3261-3267.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., Laufs, R., 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178, 175-183.

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J., Laufs, R., 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm

production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect Immun 62, 3244-3253.

Nedelmann, M., Sabotke, A., Laufs, R., Mack, D., 1998. Generalized transduction for genetic linkage analysis and transfer of transposon insertions in different *Staphylococcus epidermidis* strains. Zentralbl Bakteriol 287, 85-92.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34, 415-425.

Otto, M., 2009. *Staphylococcus epidermidis* - the 'accidental' pathogen. Nat Rev Micro 7, 555-567.

Otto, M., 2013. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity*. Annual Review of Medicine 64, 175-188.

Raad, I., Alrahwan, A., Rolston, K., 1998. Staphylococcus epidermidis: Emerging Resistance and Need for Alternative Agents. Clinical Infectious Diseases 26, 1182-1187.

Resch, A., Rosenstein, R., Nerz, C., Götz, F., 2005. Differential Gene Expression Profiling of Staphylococcus aureus Cultivated under Biofilm and Planktonic Conditions. Applied and Environmental Microbiology 71, 2663-2676.

Rogers, K.L., Fey, P.D., Rupp, M.E., 2009. Coagulase-Negative Staphylococcal Infections. Inf Dis Clin N Am 23, 73-98.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386.

Shi, L., Günther, S., Hübschmann, T., Wick, L.Y., Harms, H., Müller, S., 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry Part A 71A, 592-598.

Uçkay, I., Pittet, D., Vaudaux, P., Sax, H., Lew, D., Waldvogel, F., 2009. Foreign body infections due to *Staphylococcus epidermidis*. Ann Med 41, 109-119.

Wang, R., Khan, B.A., Cheung, G.Y.C., Bach, T.-H.L., Jameson-Lee, M., Kong, K.-F., Queck, S.Y., Otto, M., 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J Clin Invest 121, 238-248.

Yao, Y., Sturdevant, Daniel E., Otto, M., 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol soluble modulins in formation of biofilms. J Infect Dis 191, 289-298.

Zandri, G., Pasquaroli, S., Vignaroli, C., Talevi, S., Manso, E., Donelli, G., Biavasco, F., 2012. Detection of viable but non-culturable staphylococci in biofilms from central venous catheters negative on standard microbiological assays. Clin Microbiol Infect 18, E259-E261.